Amplification and Overexpression of CACNA1E Correlates with Relapse in Favorable Histology Wilms’ Tumors

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Abstract

Purpose: The most well established molecular markers of poor outcome in Wilms’ tumor are loss of heterozygosity at chromosomes 1p and/or 16q, although to date no specific genes at these loci have been identified. We have previously shown a link between genomic gain of chromosome 1q and tumor relapse and sought to further elucidate the role of genes on 1q in treatment failure.

Experimental Design: Microarray-based comparative genomic hybridization identified a micro-amplification harboring a single gene (CACNA1E) at 1q25.3 in 6 of 76 (7.9%) Wilms’ tumors, correlating with a shorter relapse-free survival (P = 0.0044, log-rank test). Further characterization of this gene was carried out by measuring mRNA and protein expression as well as stable transfection of HEK293 cells.

Results: Overexpression of the CACNA1E transcript was associated with DNA copy number (P = 0.0204, ANOVA) and tumor relapse (P = 0.0851, log-rank test). Immunohistochemistry against the protein product CaV2.3 revealed expression localized to the apical membrane in the distal tubules of normal kidney but not to the metanephric blastema cells of fetal kidney from which Wilms’ tumors arise. Nuclear localization in 99 of 160 (61.9%) Wilms’ tumor cases correlated with a reduced relapse-free survival, particularly in cases treated with preoperative chemotherapy (P = 0.009, log-rank test). Expression profiling of stably transfected HEK293 cells revealed specific up-regulation of the immediate early response genes EGR1/EGR2/EGR3 and FOS/FOSB, mediated by activation of the MEK/ERK5/Nur77 pathway.

Conclusions: These data identify a unique genetic aberration with direct clinical relevance in Wilms’ tumor relapse and provide evidence for a potential novel mechanism of treatment resistance in these tumors.

Despite the success rate of Wilms’ tumor (nephroblastoma) treatment with surgery, chemotherapy, and radiotherapy, there remains a subset of ~15% of patients who will suffer relapse, with over half dying of the disease (1). The ability to stratify treatment based on predicted response would be of immense benefit in patient management. The identification of the molecular events underpinning tumor development and relapse may also provide new targets for novel therapeutic intervention.

The search for molecular indicators of poor clinical outcome in Wilms’ tumors has focused largely on loss of heterozygosity at chromosomes 1p and 16q. The National Wilms’ Tumor Study Group-5 trial reported a relative risk of relapse for patients with favorable histology tumors, stratified by stage, of 1.56 for loss of heterozygosity at 1p (P = 0.01) and 1.49 for loss of heterozygosity at 16q (P = 0.01); loss of heterozygosity for both regions simultaneously was an even stronger predictor of outcome (2). Despite this, no individual genes on 1p or 16q have yet been shown to play a functional role in Wilms’ tumorigenesis and treatment failure.

Losses at 1p and 16q are among the most frequent regions of genomic deletion in Wilms’ tumors. Additional loci include 11p13 (WT1 locus) and 11p15 (WT2 locus) as well as 7p and 22q (3–5). Furthermore, Wilms’ tumors have been reported to harbor a variety of chromosomal aberrations resulting in gain in DNA copy number. The most frequent events include gain of the entire chromosomes 8 and 12 as well as additional whole arm gains of 7q and 1q (3–5). Of far less significance seems to be focal high-level gains or amplifications, which seem to occur only very infrequently in Wilms’ tumor genomes.

We have previously used genome-wide copy number screens to identify gain of chromosome 1q as a significant indicator...
of Wilms’ tumor relapse (6). Specifically, microarray-based comparative genomic hybridization (aCGH) revealed 1q gains to be significantly associated with a shorter time to relapse, particularly those with high-level gain, and correlated strongly with loss of 1p and/or 16q, suggesting a possible unified mechanism for these genetic alterations (7). For the most part, gain of 1q involved the whole arm, although shorter regions were also observed, with a minimal region of overlap spanning 1q21-q25. A novel breakpoint was also detected at 1p13, possibly associated specifically with isochromosome 1q formation (7). The genes spanning this minimal region on 1q have also been identified as overexpressed in relapsing Wilms’ tumors (6) and are overexpressed in those tumors with genomic 1q gain (9). Despite this, initial candidate screening has failed to identify any specific gene on 1q to be statistically differentially expressed between relapsing and nonrelapsing tumors (7, 9).

Additional mining of our genome-wide aCGH data revealed a strong association with Wilms’ tumor relapse of a recurrent microamplification of two bacterial artificial chromosome (BAC) clones with equivocal mapping information. Here, we report the definitive genomic location of these clones to 1q25.3 and the identification of alterations of a single gene, CACNA1E, at the genome, transcript, and protein levels to be associated with relapse in favorable histology Wilms’ tumors.

Materials and Methods

Sample collection. Primary Wilms’ tumor samples were obtained after approval by local and multicenter Ethical Review Committees from the North American Children’s Oncology Group and the United Kingdom Childhood Cancer Study Group. The aCGH sample set consisted of 76 favorable histology Wilms’ tumors, enriched for those that recurred, and has been described previously (7). An additional 11 samples were available for mRNA expression analysis. Samples were taken at immediate nephrectomy and frozen in liquid nitrogen. Genomic DNA was extracted using a standard proteinase K digestion followed by phenol/chloroform extraction and resuspended in water. RNA was extracted using Trizol (Invitrogen, Paisley, United Kingdom) according to the manufacturer’s instructions.

Microarray CGH and statistical analyses. The aCGH platform used in this study was constructed by the Breakthrough Breast Cancer Research Centre Microarray Facility (London, United Kingdom). Seventy-six Wilms’ tumors were analyzed on human CGH 4.6K 1.1.2 arrays (ArrayExpress accession number A-MEXP-192) consisting of 4,179 BAC clones, spaced at ~1 Mb throughout the genome as reported previously (7). All data have been submitted according to minimum information about microarray experiment guidelines (10) to the public data repository ArrayExpress with accession number E-TABM-10. Class comparison analysis was carried out using a t test adjusted for multiple testing using the step-down permutation procedure maxT (11), providing strong control of the family-wise type I error rate.

Fluorescence in situ hybridization, mapping, and end sequencing of BAC clones. Fluorescence in situ hybridization (FISH) analysis and end sequencing were carried out on interphase nuclei on tumor touch imprints as described previously (12). BAC clones RP11-46A10, RP11-_SEQ_MOVED_410D23, RP11-_SEQ_MOVED_410E24, and RP11-538D16 from the human CGH 4.6K 1.1.2 array set, RP11-814E19 and RP11-4949 from the 32K tiling path set (13), and a chromosome 1 centromere probe (Vysis, Inc., Downers Grove, IL) were labeled with either biotin or fluorescein, and the preparations were counterstained with 4’,6-diamidino-2-phenylindole in antifade (Vector Laboratories, Inc., Burlingame, CA). Images were captured using a cooled charge-coupled device camera (Photometrics, Tucson, AZ). The genomic locus of each BAC clone was determined according to the University of California at Santa Cruz BAC End Pair algorithm (8). Southern and Northern hybridizations. DNA (7 μg) was digested overnight with PstI, and fragments were resolved by 1% agarose gel electrophoresis. Total RNA (10 μg) was resolved by 1% formaldehyde-containing agarose gels. Equal sample loading in the gels was assessed by ethidium bromide staining and UV transillumination. Gels were blotted onto a nylon membrane (Hybond-N, GE Healthcare, Chalfont St. Giles, United Kingdom) and immobilized by UV cross-linking. Probes for Southern and Northern blots were PCR-amplified cDNA fragments from the COOH-terminal end of CACNA1E and labeled with [32P]dCTP using a BioPrime kit (Invitrogen) according to the manufacturer’s instructions. Hybridization was done in ULTRArhy buffer (Ambion, Huntingdon, United Kingdom) at 42°C.

Quantitative real-time reverse transcription-PCR. cDNA was prepared from 1 μg of tumor or reference RNA by random-primed reverse transcription using SuperScript II (Invitrogen). Assays-on-Demand was obtained from Applied Biosystems (Warrington, United Kingdom) for CACNA1E (Hs00167789_m1). PCRs were done in a 10 μl reaction volume containing 5 μl of 2× buffer/enzyme mix, 0.5 μl 20× assay mix, 0.5 μl 20× glyceraldehyde-3-phosphate dehydrogenase endogenous control assay mix (Hs99999905), and 1 μl input cDNA. Assays were run on an Applied Biosystems 7900 Sequence Detection System, and results were analyzed by the standard curve method. Data were normalized to human embryonic kidney HEK293 cells and the Universal Human Reference RNA (Stratagene, La Jolla, CA).

Immunohistochemistry. Immunoperoxidase labeling was done on 5-μm formalin-fixed, paraffin-embedded sections using the anti-CaV2.3 rabbit polyclonal antibody (anti-195A directed against peptide Nast-[195] of [32P]dCTP using a BioPrime kit (Invitrogen) according to the manufacturer’s instructions. Optimization experiments were carried out using frozen and formalin-fixed, paraffin-embedded sections of normal mature kidney as well as the CaV2.3-overexpressing human embryonic kidney cell line HEK2C6 (below). The primary antibody was used at a dilution in TBS of 1:1,250, and no antigen retrieval techniques were carried out. Positive (sections of frozen sections of normal kidney) and negative (omission of the primary antibody) controls were included in each slide.

Tissue microarrays. Pediatric renal tumor tissue microarrays were constructed (15) containing replicate representative cores (n = 885) from all available cellular components from 274 Wilms’ tumors, 14 clear cell sarcomas of the kidney, 9 mesoblastic nephromas, and 7 rhabdoid tumors of the kidney. Tumors were treated according to either the National Wilms’ Tumor Study Group guidelines (immediate nephrectomy) or the International Society of Pediatric Oncology protocols (delayed nephrectomy following preoperative chemotherapy). There was a slight enrichment of tumors that relapsed. The presence of tumor tissue on the arrayed samples was verified on a H&E-stained section. The intensity and subcellular localization of the Ca2+ expression was assessed independently by two pathologists (I.S.-F. and G.M.V.). Tumor cores were semiquantitatively classified according to the intensity of Ca2+ staining into three categories: negative, lack of any staining in neoplastic cells; weak/moderate, neoplastic cells with expression levels weaker than that seen in distal tubules of normal kidney; and strong, neoplastic cells expressing Ca2+ at similar levels or stronger than those seen in distal tubules of normal kidney.

Cell culture. Human embryonic kidney HEK293 cells and the CaV2.3 stably transfected HEK2C6 derivatives (16) were grown in
Fig. 1. Microamplification at chromosome 1q25.3 is associated with relapse in favorable histology Wilms’ tumors. A, Y axis, a t test for relapsing versus nonrelapsing Wilms’ tumors was calculated independently for each BAC clone. Dashed horizontal lines, max T adjusted P value of 0.05 (orange) and 0.01 (purple); vertical dotted lines, chromosome centromeres. Arrow, the most statistically significant clones are located at 1q25.3. B, log2 ratios for each clone in tumors RMH0488, RMH0516, RMH0529, and RMH3358 are plotted (Y axis) for each BAC clone according to chromosomal location (X axis). Vertical dashed lines, log2 ratios of 0.15 and −0.15. The microamplification at 1q25.3 is highlighted. C, FISH for clone RP11-___SEQ___MOVED___410D23 (red) on touch imprints for tumors RMH0516 and RMH0529 showing amplification relative to chromosome 1 centromere (green). D, FISH mapping of clone RP11-___SEQ___MOVED___410D23 confirmed location on 1q25.3. End sequencing of clones RP11-___SEQ___MOVED___410D23 and RP11-___SEQ___MOVED___410E24 showed mapping to the CACNA1E locus. E, Southern hybridization using a CACNA1E-specific probe, confirming gene amplification in Wilms’ tumors RMH0511 and RMH0516.
DMEM (bicarbonate buffered) (Sigma, Crawley, United Kingdom) supplemented with 10% FCS (BioWest, Ringmer, East Sussex, United Kingdom). All cells were incubated at 37°C with 5% CO₂. Total protein lysates (10 μg) were separated by SDS-PAGE according to standard protocols, and immunoblotting was carried out using primary antibodies directed against CaV2.3 (polyclonal anti-195A, 1:100), mitogen-activated protein kinase kinase 5 (MEK5; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), ERK5 (1:1,000), phosphorylated ERK5 Thr183/Tyr185 (1:1,000; Cell Signaling Technology, Danvers, MA), Nur77 (1:100; Santa Cruz Biotechnology), phosphorylated Nur77 Ser351 (1:100; Santa Cruz Biotechnology), and β-actin (1:2,000; Abcam, Cambridge, United Kingdom).

Expression profiling. Total RNA (10 μg) from HEK293 and HEK294 cells was used to generate labeled cRNA and hybridized to U133 Plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA) containing >40,000 transcripts according to standard manufacturer protocols. Background subtraction, normalization, and log₂ transformation of the array data were done using the Robust Multiarray Average method (17) within Bioconductor 1.5. All data have been submitted according to minimum information about microarray experiment guidelines (10) to the public data repository ArrayExpress with accession number E-MEXP-587.

Results

Microamplification at chromosome 1q25.3 is associated with relapse in favorable histology Wilms’ tumors. We did a t test, stringently corrected for multiple testing, on our aCGH to look for DNA copy number differences between favorable histology Wilms’ tumors that went on to relapse and those that did not. We observed a markedly increased test statistic for two clones mapping to 1q25.3 (maxT adjusted P < 0.001; Fig. 1A). These clones were observed as gained in 8 of 39 (20%) nonrelapsing tumors and up to 26 of 37 (70%) of relapsing cases. Investigating the individual profiles of tumors with gain of these clones, we observed that a proportion of these cases (6 of 34 with 1q gain, 17.6%; 6 of 76 overall, 7.9%) had a specific high-level gain of these clones greater than the remaining 1q BACs regardless of the copy number of the rest of the long arm (Fig. 1B). This was confirmed by interphase FISH analysis of tumor touch imprints for these and flanking sequences, which highlighted a microamplification resulting in approximately 10 to 14 copies of these clones, on a background of both disomy and trisomy 1q (Fig. 1C). The BACs RP11-SEQ_MOVED_410D23 and RP11-SEQ MOVED 410E24 were labeled as such due to the ongoing sequence validation of the array clone set reassigning their mapping from the 16p13 location designated for these clones in the genomic databases. To be certain of their localization, we FISH mapped labeled clones to metaphase chromosomes and end sequenced the DNA, confirming that the clones had identical sequence and mapped to 178,287,529 to 178,435,353 bp on chromosome 1q25.3 (Fig. 1D). The focal nature of this amplification was confirmed using BAC clones from the 32K tiling path set (data not available).

Fig. 2. CACNA1E is amplified and overexpressed in relapsing Wilms’ tumors. A. Northern hybridization using a CACNA1E-specific probe showing high mRNA expression levels in Wilms’ tumors. B. mRNA expression levels for 34 Wilms’ tumors as determined by quantitative reverse transcription-PCR. Relative expression levels are plotted on the Y axis for individual tumors along the X axis. Dark gray, relapsing tumors; light gray, nonrelapsing tumors. C. box plots comparing relative mRNA expression (Y axis) of CACNA1E in relapsing (dark gray) and nonrelapsing (light gray) tumors (X axis). D. relative mRNA expression (Y axis) of CACNA1E is plotted against 1q25.3 aCGH ratio (X axis) for each tumor. Light gray dots, tumors with increased copy number by aCGH and high-level expression by reverse transcription-PCR; dark gray dots, tumors with gain by aCGH but no corresponding gene overexpression.

shown). This region contains only a single gene, CACNA1E, which encodes the ion-conducting α1 subunit of voltage-dependent R-type calcium channels. Amplification of the gene was further confirmed by Southern hybridization (Fig. 1E).

**CACNA1E is amplified and overexpressed in relapsing Wilms’ tumors.** High levels of CACNA1E mRNA expression were observed in a number of Wilms’ tumors by Northern hybridization (Fig. 2A). Overall relative expression levels assayed by real-time quantitative reverse transcription-PCR varied up to 50-fold between the lowest and highest expressers (Fig. 2B) in a series of 34 Wilms’ tumors composed of 23 from our aCGH set and 11 independent cases (18 relapsing and 16...
nonrelapsing). Overexpression relative to normal kidney was noted in 23 of 34 (67.6%) tumors, with only two relapsing cases showing no CACNA1E transcriptional up-regulation. In class comparison analysis, there were significantly elevated levels of CACNA1E mRNA in the relapsing compared with the nonrelapsing group \( (P = 0.013, t \text{ test}; \text{Fig. 2C}) \). For the cases in which we had both copy number and expression data, we observed a significant association between CACNA1E gene copy number gain and overexpression of its mRNA \( (P = 0.0204, \text{ANOVA}; \text{Fig. 2D}) \).

CACNA1E protein expression in fetal kidney, normal mature kidney, and Wilms' tumors. We next investigated CaV2.3 protein expression by immunohistochemistry in mature normal kidney, fetal kidney, and Wilms' tumors on archival pathology specimens \( (14) \). In the fetal kidney, the developing tubules showed a positive immunoreaction localized to the cytoplasm and the cell membrane (Fig. 3A). Importantly, no staining was observed in the metanephric blastemal cells, the purported cell of origin of Wilms' tumor development (Fig. 3B). In the normal mature kidney, we confirmed previous observations reporting CaV2.3 positivity in the epithelial cells of the distal tubules (Fig. 3C; ref. 14). We noted that this was predominantly on the apical membrane but also on the basolateral membranes as well as the cytoplasm (Fig. 3D). Weak positivity could also be seen in the proximal tubules, whereas glomeruli were negative. Wilms' tumors exhibited a more complicated pattern of staining. Expression of CaV2.3 was noted in blastemal, epithelial, and stromal components. Although membranous staining was observed, this was often rather weak, with a diffusely cytoplasmic localization more apparent in the epithelial and blastemal cells (Fig. 3E). Surprisingly, we also noted a high proportion of tumors with immunoreactivity covering the nucleus. This was apparent particularly in the blastemal cells, which exhibited weak to strong staining in the nuclei, often accompanied by cytoplasmic and/or membranous localization (Fig. 3F). Although the nuclear expression was generally regarded as diffuse, not all of the cells were positive, and a heterogeneous pattern was observed for most tumors. In the epithelial cells, nuclear reactivity was less common, although once again in many tumors a heterogeneous pattern of nuclear, cytoplasmic, membranous, and negative staining was observed (Fig. 3G). In stromal cells, the expression was generally negative or weak cytoplasmic although varied with the cellular differentiation. Of note, rhabdomyoblastic cells were uniformly strongly positive in the cytoplasm (Fig. 3H).

CACNA1E is associated with shorter relapse-free survival at the DNA, RNA, and protein levels. We further sought to confirm the clinicopathologic correlations of CACNA1E/CaV2.3 in our Wilms' tumor cohorts. It is important to note that the set of patients from which frozen tumor was available for DNA and RNA analysis was enriched for relapsing cases (approximately half). Nonetheless, exploratory survival analysis showed a significantly shorter relapse-free survival time for those cases with a microamplification of CACNA1E compared with all other cases, including those with larger regions of 1q gain (Fig. 4A). The median relapse-free period in microamplification cases was 6.8 months compared with the rest of the group \( (47.8 \text{ months}; P = 0.0044, \text{log-rank test}) \). All six cases suffered a recurrence. High mRNA expression levels also predicted for tumor relapse, although this did not reach formal statistical significance \( (P = 0.0851, \text{log-rank test}; \text{Fig. 4B}) \). Only one case with low levels of CACNA1E expression suffered a recurrence.

CaV2.3 protein expression was assessed by immunohistochemistry on a pediatric renal tumor tissue microarray. Overall, in assessable cores, the vast majority \( (158 \text{ of } 189, 83.6\%) \) of favorable histology Wilms' tumors exhibited some kind of CaV2.3 positivity, with low levels of cytoplasmic reactivity evident in all cell types of most tumors. When the nuclear localization was assessed independently, positive cells were observed in 99 of 160 \( (61.9\%) \) favorable histology Wilms' tumors and also in 7 of 8 anaplastic Wilms' tumors, 11 of 12 clear cell sarcomas of the kidney, 2 of 4 mesoblastic nephromas, and 1 of 2 rhabdoid tumors of the kidneys. Our series of Wilms' tumors on the TMA were split between those treated by either preoperative chemotherapy or immediate nephrectomy, and the cases undergoing different treatment protocols were separated for survival analysis. The proportion of CaV2.3-positive tumors were higher in the group that underwent immediate nephrectomy \( (73 \text{ of } 99, 73.7\%) \) than those exposed to preoperative chemotherapy \( (38 \text{ of } 74, 51.4\%; P = 0.0037, \text{Fisher's exact test}) \). Patients treated with preoperative chemotherapy showed a significantly shorter time to relapse if their tumors exhibited any degree of nuclear CaV2.3 expression \( (\text{median survival, } 17.7 \text{ months}; P = 0.009, \text{log-rank test}; \text{Fig. 4C}) \). This was not related to an increased proportion of 'high risk' (blastemal type) tumors in the cases \( (P > 0.99, \chi^2 \text{ test}) \). An association with overall survival did not reach formal statistical significance \( (P = 0.246, \text{log-rank test}) \). There was a similar association with nuclear CaV2.3 positivity in Wilms' tumors treated with immediate nephrectomy, although this did not reach statistical significance \( (\text{median survival, } 40.8 \text{ months}; P = 0.182, \text{log-rank test}; \text{Fig. 4D}) \). There was no association with overall survival in these cases \( (P = 0.473, \text{log-rank test}) \). Multivariate analysis using a Cox proportional hazards model fitted with tumor stage, age at diagnosis, and CaV2.3 nuclear positivity revealed a borderline significance as an independent prognostic factor for relapse-free survival for the preoperative chemotherapy group \[ \text{hazard ratio, } 2.10 \text{ (SE, } 0.386); P = 0.055, \text{Cox proportional hazards}] \) and no association in the immediate nephrectomy cases \[ \text{hazard ratio, } 1.13 \text{ (SE, } 0.355); P = 0.74, \text{Cox proportional hazards}] . Further analysis of clinicopathologic variables failed to reveal any significant correlations of CaV2.3 expression with age at diagnosis \[ (P = 0.644, \text{Mann-Whitney U test}) \] or tumor stage \[ (P = 0.588, \chi^2 \text{ test}) \].

CaV2.3-overexpressing human embryonic kidney cells have an activated MEK5/ERK5 pathway and up-regulate the early response genes EGR1/EGR2/EGR3 and FOS/FOSB. To investigate the mechanisms by which CACNA1E up-regulation may be playing a role in Wilms' tumor progression, we carried out expression profiling of human embryonic kidney HEK293 cells stably expressing CaV2.3 \( (18) \) using Affymetrix U133 Plus 2.0 arrays. There was a significant up-regulation of the immediate early response genes EGR1/EGR2/EGR3 and FOS/FOSB in the CaV2.3-overexpressing cells (Table 1). We also observed overexpression of MAP2K5 \( (\text{MEK5}) \) and NR4A2 \( (\text{Nur77}) \), which form part of the MEK5/ERK5/Nur77 signal transduction pathway thought to up-regulate early response genes, such as EGR and FOS \( (19) \). To determine whether this pathway was specifically activated in the CaV2.3-overexpressing cells, we investigated the protein expression of this pathway by
Western blot analysis. Although we could detect no difference in total MEK5 levels, we observed increased expression of total and phosphorylated ERK5 and total Nur77 in the CaV2.3-overexpressing cells (Fig. 5).

**Discussion**

Recurrent gene amplifications are not a reported feature of Wilms’ tumor genomes as have been shown in a variety of other cancers, including both pediatric and adult tumor types (20). Our initial aCGH study of favorable histology Wilms’ tumors confirmed, in most cases, the large regions involved in many of the observed chromosomal aberrations, although previously unreported focal changes were seen (e.g., gains of 15q and losses of 10p) as well as definition of high-level regions of copy number increase on chromosomes with frequent whole arm gain (e.g., 8p and 12p; ref. 7).

One such region is chromosome 1q, gain of which is associated with Wilms’ tumor relapse (6, 7). We identified a recurrent microamplification of two clones, end sequenced, and FISH mapped to reveal that they were identical and had a genomic location within the 1q minimal region of overlap specifically at 1q25.3. These clones mapped to a single gene, **CACNA1E**, as defined by the end and start locations of the flanking clones on the array platform. The focal nature and the high level of the amplification were confirmed by in situ hybridization of BAC clones from our array platform as well as the 32K tiling path set (13). Thus, we have identified a gene-specific amplification in Wilms’ tumor and showed that this may be present on the background of normal copy number of the rest of chromosome 1q or low-level gain of the whole arm. **CACNA1E** amplification was found to confer an increased rate of and shorter time to relapse than normal copy number or whole arm gain. Increased copy number was directly related to a higher mRNA expression of the gene, which was itself associated with relapse.

**CACNA1E** encodes the ion-conducting α₁ subunit of R-type voltage-dependent calcium channels widely expressed throughout the brain as well as in endocrine systems (18) and has not previously been implicated in tumorigenesis. Recent reports, however, showing the expression of splice variants in the distal tubules of human kidney (14) reveal a potential role in
nephrobiology. In particular, it has been suggested that the encoded protein CaV2.3 may be involved in the modulation of Ca\(^{2+}\)-mediated hormone secretion (18, 21).

Immunohistochemical staining using an antiserum raised against CaV2.3 confirmed the distal tubule expression in normal mature kidney cells and furthermore showed a preferential staining at the apical surface of the cell membrane. It is unclear whether this apical localization is related to the mediation of calcium entry or potential secretory functions. In the fetal kidney, CaV2.3 expression was noted to have a similar pattern in the epithelial cells of the developing tubules but was entirely absent from the metanephric blastema from which Wilms' tumors are thought to arise. It is potentially of significance, whether this apical localization is related to the mediation of calcium entry or potential secretory functions. In the fetal kidney, CaV2.3 expression was noted to have a similar pattern in the epithelial cells of the developing tubules but was entirely absent from the metanephric blastema from which Wilms' tumors are thought to arise. It is potentially of significance, whether this apical localization is related to the mediation of calcium entry or potential secretory functions.

Voltage-gated Ca\(^{2+}\) channels mediate the entry of Ca\(^{2+}\) into cells in response to membrane depolarization (23). The Ca\(\alpha_2\) family of \(\alpha_1\) subunits initiates rapid synaptic transmission and is regulated primarily by direct interaction with G proteins and SNARE proteins and secondarily by protein phosphorylation (23). The CaV2.3 subunit forms part of the R-type high voltage-activated Ca\(^{2+}\) channel, which is distinguished by its insensitivity to \(\omega\)-agatoxin-IVA and \(\omega\)-conotoxin-GVIA. There has been an increasing interest in calcium channels and cancer, as Ca\(^{2+}\)-mediated intracellular signaling pathways control a large range of cellular processes, including proliferation (24). The identification of up-regulation of other calcium channel subunits in colon (22) and prostate (25) cancer suggests a potential generalized link with tumorigenesis, although the mechanisms by which this occur remain unclear.

Stable transfection of CaV2.3 into human embryonic kidney cells resulted in the specific activation of the MEK5/ERK5/Nur77 pathway, leading to up-regulation of the proliferation-associated

**Table 1. Affymetrix expression profiling of CaV2.3-overexpressing human embryonic kidney cells**

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NOTE: Transcripts overexpressed > 1.5-fold (log\(_2\)) in CaV2.3-overexpressing HEK293 cells after preprocessing using the Robust Multiarray Average package.
poorly understood although there are thoughts to have diverse effects on cellular proliferation, migration, invasion, and induction of protease production. Of particular interest is the enhancement of activator protein-1 activities in prostate cancer cells by MEK5 activation (28). Specific up-regulation of FOS (19) and FOSB is of potential translational significance, as these genes were found to be specifically overexpressed in CACNA1E-amplified versus nonamplified Wilms’ tumors by cDNA microarray analysis. A further intriguing observation is the up-regulation of various Frizzled genes by CaV2.3 overexpression (both in HEK293 cells and Wilms’ tumors), hinting at a role for Wnt/Ca2+ pathways (in addition to the previously implicated canonical pathways) in Wilms’ tumors.

Although much remains to be done to elucidate the mechanisms by which amplification and overexpression of a calcium channel subunit may play a role in Wilms’ tumorigenesis and/or chemoresistance, there exists the possibility of a novel therapeutic intervention strategy. The CaV2.3 channels are blocked specifically by the synthetic peptide toxin SNX-482 derived from tarantula venom, and the same CaV2.3-overexpressing HEK293 cells used in this study have previously been shown to be blocked at micromolar concentrations by a range of structurally diverse carbonic anhydrase inhibitors (29). Further functional and preclinical evaluation will be necessary to translate our unique molecular genetic finding into clinical application for relapsed Wilms’ tumor patients.

Fig. 5. Activation of the MEK5/ERK5 pathway in CaV2.3-overexpressing human embryonic kidney cells. Western blot analysis shows a specific activation of the MEK5/ERK5/Nur77 signal transduction pathway in human embryonic kidney cells in response to stable transfection with CaV2.3.

References

stress-induced hyperglycemia upon disruption of the Ca(v)2.3 (α1E) subunit of voltage-gated Ca(2+) channels. Mol Endocrinol 2002;16:884–95.


Amplification and Overexpression of CACNA1E Correlates with Relapse in Favorable Histology Wilms' Tumors

Rachael Natrajan, Suzanne E. Little, Jorge S. Reis-Filho, et al.


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